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# **ANNUAL TECHNICAL REPORT**

**Title:** Hepatic Metabolism of Perfluorinated Carboxylic  
Acids: A Nuclear Magnetic Resonance Investigation

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## ABSTRACT

This research project employs nuclear magnetic resonance (NMR) spectroscopy to investigate the metabolic aspects of the toxicity associated perfluorinated carboxylic acids. Fluorine-19 NMR has been used to monitor the metabolic fate of perfluoro-n-octanoic acid (PFOA) and perfluoro-n-decanoic acid (PFDA) in the rat. Spectra obtained at various times following the administration of PFOA or PFDA depict the presence of the parent compounds in samples of bile, serum, urine, and liver *in vivo*. Urine spectra also indicate the presence of a possible metabolite which has not been identified at this time. Carbon-13 NMR is providing information regarding the effects of PFOA and PFDA on hepatic carbohydrate metabolism. Preliminary data indicate that hepatic glycogenesis is severely inhibited in rats at  $\geq 3$  days post treatment with PFDA. Plasma glucose and hepatic glucose appear to behave similar to control animals during the first three days post treatment with PFDA, but data obtained at days 6 and 7 indicate that hepatic glucose utilization may be slowed. These data are preliminary and experiments are currently in progress to further characterize the perfluorocarbon-induced dysfunctions of liver metabolism.



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## **Annual Technical Report**

### **INTRODUCTION**

The primary objectives of this research project are to evaluate the utility of nuclear magnetic resonance (NMR) techniques in toxicological studies and to specifically investigate the metabolic effects caused by perfluoro-n-octanoic acid (PFOA) and perfluoro-n-decanoic acid (PFDA) in rats.

During the past 10 month period our efforts have focused on two areas of study: (i) the use of  $^{19}\text{F}$  NMR to monitor the distribution and metabolic fate of fluorocarbon compounds in rat liver and bodily fluids and (ii) the use of  $^{13}\text{C}$  NMR to monitor the effects of PFDA on hepatic carbohydrate metabolism *in vivo*. This report will provide a synopsis of the results and accomplishments of this work. The  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR studies will be discussed separately.

### **I. FLUORINE-19 NMR STUDIES**

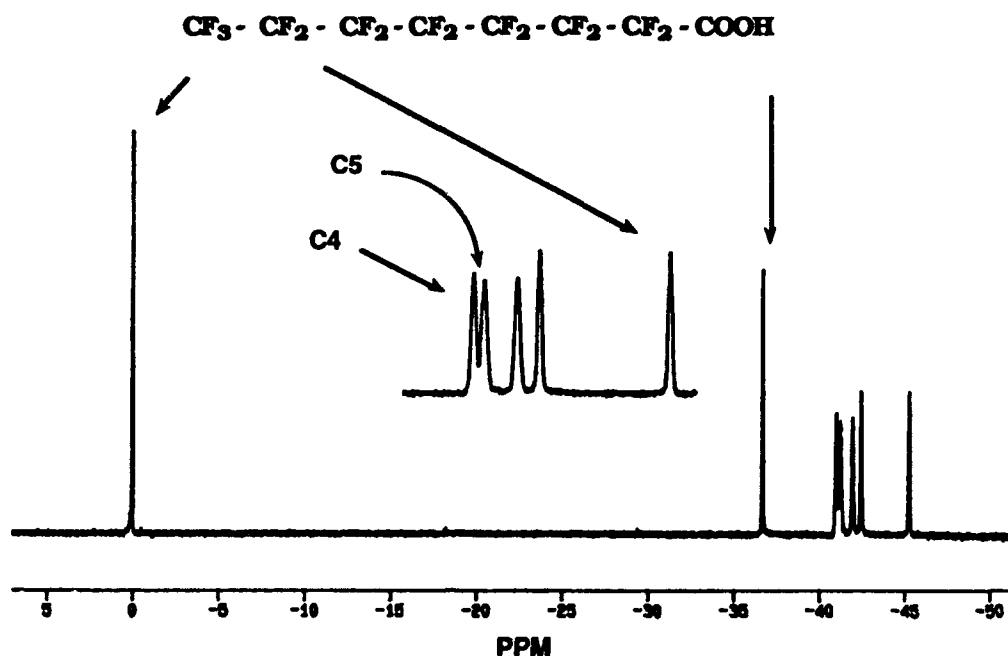
Since the commencement of this project in February 1990, our laboratory has been engaged in a fluorine-19 NMR investigation of the toxicology associated with PFDA and PFOA in rats. The focus of these studies, which are still ongoing, is to characterize the  $^{19}\text{F}$  NMR spectrum of these compounds and determine if metabolites of these perfluorinated carboxylic acids can be detected in spectra obtained from the rat *in vivo*. These research efforts also provide an opportunity to evaluate the utility of  $^{19}\text{F}$  NMR for toxicological investigations of fluorocarbon compounds.

The  $^{19}\text{F}$  NMR experiment provides a fingerprint of molecular structure, thus, the metabolic transformation of fluorocarbon compounds will be evident in the NMR spectrum. The detection of these compounds in liver *in vivo* and from various body fluids from the rat has been accomplished and the results are outlined below.

### **Results and Discussion**

Figure 1 shows a high-resolution  $^{19}\text{F}$  NMR spectrum of PFOA obtained with a Bruker AM 360 NMR spectrometer at 8.5 tesla (T). A two-dimensional correlation spectroscopy (2D-COSY) experiment was performed in order to identify the spectral assignments indicated at the top of the spectrum. The resonances of the fluorines on carbons C3 and C6 were not completely

resolved in the 2D experiment and, therefore, these assignments remain ambiguous. Spin-lattice relaxation times ( $T_1$ ) for specific resonances were measured by the inversion-recovery technique and are listed in Table 1.



**Figure 1.** A  $^{19}\text{F}$  NMR spectrum of 50 mM PFOA in propylene glycol/ $\text{H}_2\text{O}$  (1:1, v/v) at 8.5 T and 27° C. The insert is an expansion of the low-frequency region between -40 and -47 ppm. The spectrum was processed with 32 K total data points and represents 5 minutes of signal averaging. Spectral assignments were determined by a 2D-COSY NMR experiment and chemical shifts are relative to the  $\text{CF}_3$  resonance which was set at zero ppm.

### NMR Analysis of Liver *in Vivo*

With the use of a surface coil probe (constructed in-house) a  $^{19}\text{F}$  NMR spectrum was obtained from the liver *in vivo* in a completely noninvasive manner such that individual animals were monitored by this technique for several days following treatment with PFOA or PFDA. A rat was given a single intraperitoneal (ip.) injection of 50 mg/kg PFOA in propylene glycol/ $\text{H}_2\text{O}$

(1:1, v/v) and  $^{19}\text{F}$  NMR spectra were obtained at 9 and 13 days post treatment. Another animal was administered the same dose of PFDA and spectra were obtained at 2 and 3 hours post treatment, and also at 2, 3, 7, 9, 10, 13, 22 and 51 days after treatment.

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**Table 1**

**Fluorine-19  $T_1$  of PFOA at 8.5 tesla**

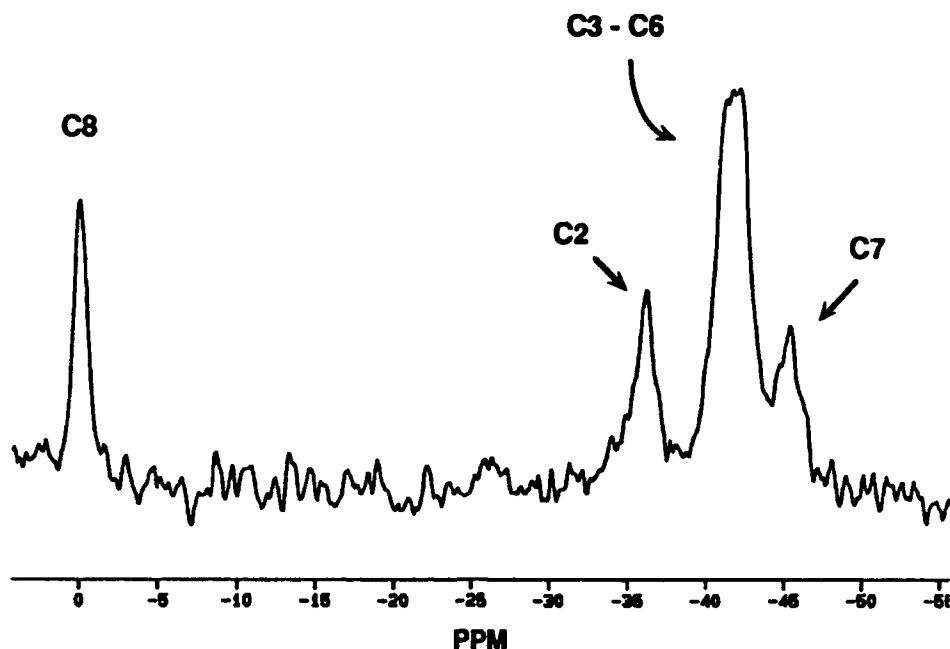
<b>*<math>^{19}\text{F}</math> on carbon N</b>	<b><math>T_1</math> (sec)</b>
C2	0.80
C4	0.83
C5	0.83
C7	0.85
C8	0.91

\*Fluorines are designated by the carbon atom to which they are chemically bonded.

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Figure 2 depicts the  $^{19}\text{F}$  NMR spectrum at 2.35 T (Biospec System) obtained from the rat liver *in vivo* at 13 days post ip. injection of PFOA. This spectrum is obviously of lower resolution than that shown in Figure 1 due to the decrease in magnetic field strength and increase in linewidths which are typical of *in vivo* spectra. Even though the rat liver spectrum is lower in resolution, the characteristic features of the PFOA spectrum are clearly discernible suggesting that only the parent compound is present in the liver of this animal. In fact, in all the spectra from liver *in vivo* only resonances corresponding to the parent compound are observable. The results indicate that either: (i) PFOA and PFDA are not metabolized in liver, or (ii) any metabolites that are formed from these compounds are not observable by NMR. Such metabolites of these perfluorinated carboxylic acids may either be present at concentrations below the limits of detection of  $^{19}\text{F}$  NMR (estimated to be a few millimolar), or otherwise they may be bound to

macromolecules with highly restricted molecular motions, rendering these compounds "NMR invisible".



**Figure 2.** A  $^{19}\text{F}$  surface coil NMR spectrum at 2.35 T of the rat liver *in vivo* acquired at 13 days post treatment with PFOA (50 mg/kg ip.). The spectral assignments are designated by specifying the carbon atom to which the fluorines are chemically bonded. This spectrum represents 25 minutes of signal averaging and the free induction decay signal was processed using 2 K total data points and a 10 Hz exponential filter. Chemical shifts are relative to the  $\text{CF}_3$  resonance (carbon C8) which is set at zero ppm.

### NMR Analysis of Bodily Fluids

Samples of urine, bile, and serum were also obtained for NMR analysis from rats at various days post treatment with PFOA or PFDA, and at various doses ranging from 5 to 100 mg/kg. In general, the spectra indicate that the parent compound is present in all these bodily fluids; however, PFDA appears to be more soluble in bile than in urine while the opposite is true for PFOA. This conclusion is based upon the signal intensity obtained in the various spectra. This finding is not surprising since PFOA is more water soluble than PFDA and has been found to be more readily excreted in the urine (1).

In another experiment, livers were excised from rats three days post administration of PFOA (100 mg/kg) or PFDA (50 and 100 mg/kg). These livers were homogenized and placed into a 5 mm NMR tube for  $^{19}\text{F}$  NMR analysis at high field (8.5 T). In addition, livers were fractionated and samples of mitochondria, cytosol, and microsomes were also analyzed using  $^{19}\text{F}$  NMR. Although the signal-to-noise ratio in the NMR spectra of these specimens was quite poor, the parent compound was revealed.

The most interesting finding of these studies relates to the presence of an NMR signal which has not yet been identified. In the urine spectra from both PFOA and PFDA-treated animals, there is a single peak at 37 ppm (relative to the chemical shift scale depicted in Figures 1 and 2) which cannot be attributed to the parent compounds. This peak may be from a metabolite which is eliminated through the urine.

### **Conclusions**

In summary, these studies demonstrate that both PFOA and PFDA can be observed in the rat liver *in vivo* and in various body fluids by  $^{19}\text{F}$  NMR spectroscopy. The results indicate that these perfluorinated carboxylic acids persist in the animal as the parent compound for many days and corroborate the findings of Ylinen, et al. (1) and Olson et al. (2). The  $^{19}\text{F}$  NMR spectrum identifies the parent compound and shows a slow decline in the signal intensity over time which is indicative of a decrease in concentration. In addition, the data show no detectable fluorinated metabolite from PFOA or PFDA in liver, bile, or serum; however, urine spectra do reveal the presence of a possible metabolite. Experiments are in progress to further investigate this finding in terms of identifying the compound or compounds which are present in the urine from PFOA and PFDA-treated rats.

## **II. CARBON-13 NMR STUDIES**

The effects of PFOA and PFDA treatment on hepatic glucose and glycogen metabolism is being investigated by proton-decoupled  $^{13}\text{C}$  NMR spectroscopy. Serial NMR spectra are obtained from liver *in vivo* while the animal is administered  $^{13}\text{C}$  labeled glucose. Plasma glucose is measured at various times during the course of the experiment to complement the NMR data.

It should be noted that these studies are currently ongoing and, to date, data has only been obtained for a few PFDA-treated animals and controls. The data are preliminary at this time

and, therefore, any inferences drawn from these data are somewhat premature and may not be conclusive.

## **Methods**

Rats are administered an intraperitoneal injection of PFDA (50 mg/kg) and  $^{13}\text{C}$  NMR data are obtained at 1, 2, 3, 5, and 8 days post treatment. A control group is given an equal volume of vehicle (1: 1 v/v propylene glycol/water) and their daily allotment of food is equal to that which is consumed by the experimental animals (pair-feeding).

The NMR studies involve surgical exposure of the liver and the use of a double resonance surface coil probe to obtain proton-decoupled  $^{13}\text{C}$  spectra from liver *in vivo* (3). A catheter is placed into the femoral vein of each leg for the administration of substrates and periodic withdrawal of blood.

The NMR experiments employ a Bruker AM 360 NMR spectrometer operating at a centerband frequency of 90.6 MHz and spectra are obtained with five minute time resolution. Four baseline spectra are acquired prior to a bolus administration of 0.6 g/kg [ $1-^{13}\text{C}$ ] glucose (99% atom enriched) via the right femoral vein catheter. Carbon-13 spectra are continually acquired for an additional two hours following the glucose injection.

Blood samples (200  $\mu\text{l}$ ) are withdrawn from the left femoral vein before the administration of glucose (baseline) and at 5, 15, 30, 60, 90, and 120 min. post glucose dose. The samples are immediately centrifuged and the plasma is frozen for subsequent glucose analyses. At the completion of the NMR experiment a sample of urine is also obtained from the bladder for glucose analysis. These analyses are being conducted at the H.G. Armstrong Aerospace Medical Research Laboratory, Wright Patterson Air Force Base, Ohio, using a Kodak Ektachem 700 XR Analyzer.

## **Results and Discussion**

The C1 carbon of both glucose and glycogen are well resolved in the  $^{13}\text{C}$  NMR spectrum and, thus, this methodology enables the simultaneous measurement of hepatic glucose and glycogen stores (4). In normal rats an exogenous glucose load is rapidly incorporated into hepatic glycogen (4). This is also the case for the pair-fed control animals used in the present study. In contrast, PFDA-treated rats show a much reduced production in glycogen at 24 hours post

treatment, and a complete inhibition of glycogen synthesis at  $\geq 3$  days post treatment.

Plasma glucose data has only been obtained from animals at 1, 2, and 3 days post-dose with PFDA. These data indicate that the experimental animals behave in a similar fashion to the control group. The glucose concentration rises due to the exogenous glucose load and then returns to baseline values by ca. 30 minutes post dose. The rate of utilization of hepatic glucose (as measured by  $^{13}\text{C}$  NMR) also appears to be normal in the PFDA-treated animals at 1, 2, and 3 days post dose; however, data obtained from rats at days 6 and 7 post treatment suggest that hepatic glucose utilization may be slowed. All urine specimens obtained at the completion of the NMR experiment (120 min. post glucose) show exceptionally high concentrations of glucose ( $\geq 200$  mg/dl).

These preliminary data suggest that PFDA causes an acute inhibition of hepatic glycogenesis. Surprisingly, the rate of disposal of both hepatic glucose and blood glucose appears to be unaffected up to 3 days post treatment. The high glucose levels of the urine indicate that the kidneys are functioning to remove the excess blood glucose.

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## **ABSTRACTS/PRESENTATIONS**

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